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Docket No. 20164 (C38435/109730) PATENT APPEAL

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

Monika JOHANNSEN

Serial No.: 09/335,022

Filed: June 17, 1999

For: PROCESS FOR PRODUCING VITAMIN D_3 AND PREVITAMIN D_3

Examiner: Sabiha Naim Qazi

Group Art Unit: 1616

New York, New York
July 15, 2002

APPELLANT'S BRIEF ON APPEAL

Commissioner for Patents
Washington, D.C. 20231

Sir:

This is an appeal from the final rejection of all claims which are pending in this application.

In accordance with 37 CFR § 1.192(a), this brief is being submitted in triplicate, together with a check in the amount of $320.00 in payment of the fee required upon filing this brief. 37 CFR § 1.17(c).

Since the Notice of Appeal was accorded a filing date of March 13, 2002, a two month extension of time is hereby requested and a check in the amount of $400.00 in payment of the fee required for the extension also is submitted herewith. 37

CFR § 1.17(a)(2). Accordingly, this brief is filed timely upon mailing, with an executed Certificate of Mailing, on or before July 15, 2002, since July 13 fell on a Saturday. 35 USC § 21(b); 37 CFR §§ 1.7(a), 1.8, 1.136, and 1.192(a).

IDENTIFICATION OF REAL PARTY IN INTEREST

The real party in interest is ROCHE VITAMINS INC., which is the assignee of record of the present application and which is a corporation organized and existing under and by virtue of the laws of the State of Delaware. Ownership of ROCHE VITAMINS INC. lies in F. HOFFMANN-LA ROCHE AG., a company organized and existing under the laws of the Swiss Confederation.

RELATED APPEALS AND INTERFERENCES

Upon information and belief of the undersigned counsel, appellant and the assignee of record are not aware that there are any pending appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

STATUS OF ALL CLAIMS AND AMENDMENTS

A. Status Prior to Final Rejection

As filed, this Rule 53 Continued Prosecution Application contained claims 1-8. In connection with the filing of this application, claim 1, the only independent claim, was amended to recite limitations that had been presented by amendment after final rejection in the parent of this application, but were not entered in that application. In addition, in a Preliminary Amendment that accompanied the filing of this application,

claim 1 was further amended to recite that the claimed process is carried out by normal phase chromatography.

No further amendments were presented prior to final rejection.

B. Status After Final Rejection

Amendments were presented to the claims and to the specification after final rejection, but were not entered.

C. Identification Of Claims On Appeal

Claims 1-8 are on appeal and are reproduced in the APPENDIX to this brief.

SUMMARY OF THE INVENTION AND THE CLAIMS

The D vitamins are biologically active substances that are essential for the regulation of calcium metabolism in higher animals. The various D vitamins differ by the nature of the side chain, and, generally, the most important members of the D vitamins are vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). The D "previtamins" also are widely distributed in higher animals and plants. Today, the industrial production of the D vitamins is carried out by the conversion of natural precursors, which are related to cholesterol. (Spec., p.1, lns. 8-16).

Vitamin D_3 is sensitive to light, air, heat, and acid, and is insoluble in water, difficultly soluble in fatty oils and has good solubility in ethanol, chloroform, ether and acetone. The melting point of vitamin D_3 lies in the range from 84 to 87°C, and it is known that the solubility of D vitamins in supercritical or subcritical fluids, *e.g.* in

supercritical CO_2, is in the temperature range from 40 to 60°C and a pressure range from 20 to 35 MPa. (Spec., p.1, lns. 17-27).

Industrial processes for the synthesis of vitamin D_3 are based on the irradiation of 7-dehydrocholesterol ("DHC"), which is produced from cholesterol. DHC, in turn, is converted by irradiation into previtamin D_3, which is then isomerized to vitamin D_3 by gentle heating. However, undesired side products, such as lumisterol and tachysterol, also are formed when DHC is irradiated. Thus, the yield of previtamin D_3, and consequently of vitamin D_3, depends essentially on the irradiation conditions. To reduce the unwanted side products that result from the irradiation, various processes are used for the purification of the mother liquor at the conclusion of the irradiation. For example, the undesired tachysterol has been converted using a Diels-Alder reaction into a tachysterol di-K salt adduct, and the salt adduct was subsequently separated. (Spec., p. 1, ln. 21, to p. 2, ln. 4).

The conventional processes have a number of disadvantages. For example, the vitamin D_3 yield is limited by the state of equilibrium in the irradiation reaction. In addition, the performance of the Diels-Alder reaction requires additional chemicals and does not give a complete yield of vitamin D_3 or previtamin D_3 based on the crude product. And purification to crystalline grade also requires additional reactions using chemicals such as pyridine and butyryl chloride but, again, no complete reaction takes place. Thus, the processes conventionally employed in the art result in losses of the valuable product. (Spec., p. 2, lns. 5-10)

Accordingly, the present invention provides a process that accomplishes the isolation of vitamin D_3 or previtamin D_3 from a mixture containing vitamin D_3 or

previtamin D_3 while avoiding the drawbacks associated with the conventionally employed processes noted above. The present invention accomplishes this by separating vitamin D_3 or previtamin D_3 from a mixture containing the vitamin or previtamin through the use of normal phase column chromatography wherein the mobile phase contains supercritical carbon dioxide or liquid carbon dioxide. (Claims 1, 7 and 8). (Spec., p. 1, lns. 3-6; and p. 2, lns. 11-18).

The mobile phase may further include a modifier (claim 2), and the stationary phase may be a silica gel (claim 3). (Spec., p. 2, lns. 16-18). More particularly, when the stationary phase is silica gel, it is in the form of homogeneously packed, spherical particles having a particles size of about 5 to 25 μm. (Claim 4; spec., p. 4, lns. 24-30; and see claim 4 as filed).

The reaction mixture that is passed through the column may be synthetically produced by irradiation and contain a mixture of vitamin D_3 isomers. (Claim 5; spec., p. 2, lns. 11-13; and p. 2, ln. 25, to p. 3, ln. 5; and see claim 5 as filed). Preferably, the process is carried out in the temperature range from about 30°C to about 60°C and in the pressure range from about 7.0 to about 15.0 MPa. (Claim 8; spec., p. 5, lns. 6-7).

STATEMENT OF THE SOLE REJECTION AND ISSUE

Whether all claims are unpatentable under 35 USC §103(a) over Zhang *et al.*, HPLC determination of vitamin D preparation, HCAPLUS ACS Abstract Document No. 113:198104 (abstract of Zhongguo Yiyao Gongye Zazhi (1990), 21(6), 256-61)) ("Zhang") in view of Lee *et al.*, Application of reversed-phase high performance liquid chromatography using enhanced-fluidity liquid mobile phase, HCAPLUS ACS Abstract

Document No. 124:105153 (abstract of J. Microcolumn Sep. (1995), 7(5), 477-83)) ("Lee").

GROUPING OF CLAIMS

Not all claims stand or fall together.

Arguments are presented below which demonstrate the patentability of claims 1-3, 5, and 8.

Separate arguments are presented which demonstrate the separate patentability of claims 4, 6, and 7.

SUMMARY OF THE DISCLOSURES OF THE REFERENCES

In view of the brevity of the Zhang and Lee abstracts, the entirety of each abstract is reproduced below.

The Zhang abstract is entitled "HPLC determination of vitamin D preparation," and it discloses:

> In the column system suitability test following irradn. of heated vitamin D_3 soln. by UV with main wave length 254 and 365 nm for 5 min, 6 isomers were sepd. with the normal-phase HPLC (Waters Resolve Silica column, 0.3% n-pentanol in hexane as mobile phase, detected at 254 nm), with resoln. factor, R, >1.0. The linearity was obtained in 0.5-60 .mu.g vitamin D_3. The low concn. (1ppm) prepn. could be detd. by internal (di-Me phthalate) method or external (thermal equil.) method with error 10%. The error in detn. of high-concn. prepn. was 3%.

The Lee abstract is entitled "Applications of reverse-phase high performance liquid chromatography using enhanced-fluidity liquid mobile phases," and it discloses:

> Enhanced-fluidity liq. mobile phases (methanol/H2O/CO2) were used as eluents in reversed-phase HPLC. The low

pressure drop across the column allowed serial connection of micro-scale columns to achieve the efficient sepn. of a coal tar sample. Other applications such as the sepn. of fat sol. vitamins and probucol and related compds. are shown.

SUMMARY OF THE POSITIONS TAKEN BY THE EXAMINER IN THE FINAL ACTION

In the Final Action, the Examiner rejected claims 1-8, presumably for the reasons asserted in the Paper No. 15 Office action, and advanced further new arguments.[1/] (Paper No. 17, pp. 2 and 3).

In the Paper No. 15 Office Action, the Examiner relied on the Zhang abstract for "teach[ing] the separation of 6 isomers separated by using irradiation technique and the purification on silica column (stationary phase)." (Paper No. 15, p. 3).

The Examiner acknowledged, however, that Zhang failed to disclose "liquid CO2 for separation by column chromatography using liquid CO2" as recited by claims 1-6 and 8. (Paper No. 15, p. 3).

To fill the acknowledged gap, the Examiner relied on the Lee abstract to "alleviate this deficiency by teaching the separation of coal tar vitamins and other related compounds. The enhance fluidity liquid mobile phase containing CO2/methanol/water [*sic*: "methanol/H_2O/CO_2"] are used in a column [*sic*: "reverse-phase HPLC"]." (Paper No. 15, p. 3).

1/ In the Final Action (Paper No. 17), the Examiner merely identified the references of the rejection without providing any basis in fact or law to support the final rejection. Thus, it is presumed that the Examiner relied on the reasoning advanced in the Paper No. 15 Office Action. If this understanding is incorrect, the Examiner is requested to withdraw the final rejection and indicate the precise reasoning relied upon in support of the rejection.

With regard to the abstracts relied upon, the Examiner contended that it would have been obvious to combine them "to separate vitamin D derivatives," arguing:

> It would have been obvious to one skilled in the art to combine the teachings of prior art supra to separate the vitamin D derivatives particularly when Zhang et al. teaches irradiation technique and the purification on silica column (stationary phase) and Lee et al. Teaches the use of liquid CO2 for separation. (Paper No. 15, p. 4, lns. 1-6).

With regard to the factual question of motivation to select and combine Zhang and Lee, the Examiner merely relied on the "reasons" previously stated, arguing:

> One skilled in the art would find ample motivation from the prior art to separate vitamin D derivatives as instantly claimed for the reasons cited above. (Paper No. 15, p. 4, lns. 7-9).

Finally addressing the claimed process, the Examiner contended that:

> Nothing unobvious to separate the vitamin D as instantly claimed by the process known in the art, unless any unexpected results are seen. (Paper No. 15, p. 4, lns. 11-12).

With regard to dependent claims 3 and 4, the Examiner summarily contended that the limitations required by those claims "would have been within the skills of the one familiar with the art," and that the "selection of" size and shape of silica gel "would be no problem for who is familiar with the separation techniques such as HPLC and column chromatography." (Paper No. 15, p. 4, lns. 16-21).

In the Final Action, in response to appellant's showing that:

> (i) The Examiner's reasoning for why one would have selected and combined Zhang and Lee -- that it would have been "obvious to combine" them because "Zhang teaches irradiation technique and purification on silica column (stationary phase) and Lee teaches the use of liquid CO_2 for separation" -- simply was not the standard, was insufficient to fill the suggestion

and motivation gap, and inaccurately characterized Lee's disclosure;

(ii) Contrary to the Examiner's broad brush interpretation of Lee, Lee did not disclose the use of a methanol/H_2O/CO_2 mobile phase "in a column," but rather specifically in ***reverse-phase*** HPLC;

(iii) The Examiner had only selected so much of the Lee abstract as would support her rejection and did not consider factual disclosure in Lee that refuted the rejection; and

(iv) The Lee abstract was an incomplete representation of the factual disclosure of the entire Lee article, and that the Lee article's disclosure, that ***reverse-phase*** HPLC was the only way to resolve vitamin D_2 and D_3 from a mixture of fat soluble vitamins, lead away from Zhang's normal phase HPLC;

the Examiner summarily contended that the "instant invention does not give any separation of vitamin D_3 and vitamin D_2 by normal phase HPLC, rather it is claiming separation of vitamin D_3 with other components such as dehydrocholesterol, lumisterol and others." (Paper No 17, p. 2, ¶ 2, Ins. 2-8).

THE LEGAL STANDARD

To reject claims in an application under 35 USC § 103, an examiner must show an unrebutted *prima facie* case of obviousness. *See In re Deuel*, 34 USPQ2d 1210, 1214 (Fed. Cir. 1995). Obviousness must be based upon facts. *Ex parte Saceman*, 27 USPQ2d 1472, 1474 (BPAI 1993). When a conclusion of obviousness is not based on facts, it cannot stand. *Ex parte Porter*, 25 USPQ2d 1144, 1147 (BPAI 1992). Suggestion and motivation must be based on "actual evidence" that must be

"clear and particular." *In re Dembiczak*, 50 USPQ2d 1614, 1617 (Fed. Cir. 1999). "When the PTO asserts that there is an explicit or implicit teaching or suggestion in the prior art, it must indicate where such a teaching or suggestion appears in the references." *In re Rijckaert*, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993) (citing *In re Yates*, 211 USPQ 1149 (CCPA 1981)). In the absence of a proper *prima facie* case of obviousness, an applicant who complies with the other statutory requirements is entitled to a patent. *See In re Oetiker*, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). On appeal to the Board, an applicant can overcome a rejection by showing insufficient evidence of *prima facie* obviousness. *See id.*

SUMMARY OF THE EVIDENCE RELIED UPON BY APPELLANT

Appellant relies on Lee *et al.*, Applications of Reversed-Phase High Performance Liquid Chromatography Using Enhanced-Fluidity Liquid Mobile Phases, *J. Microcolumn Separations*, 7(5) pp. 477-483 (1995) (Exhibit 1) (the "Lee article"), which is the full article from which the Lee abstract was cropped, and which was made of record October 3, 2000.

The Lee article discloses five reversed-phase HPLC analyses performed on various samples, wherein mobile phase mixtures employed in the analyses were methanol/H_2O (referred to as room temperature ("RT") and elevated temperature ("ET") mobile phases) and a methanol/H_2O/CO_2 mixture (referred to as enhanced fluidity ("EF") or elevated temperature-enhanced fluidity ("ET-EF") mobile phases). (P. 478, left col., lns. 1-20). Three of the five analyses were performed on a "coal tar" mixture, which was the "primary" sample for the study. The other two analyses were performed on probucol, and a mixture of vitamins that included vitamin A, BHT, vitamin A

aldehyde, vitamin D_2, vitamin D_3, vitamin A acetate, vitamin E, vitamin E acetate, and vitamin K_1. (P. 477, rt. col., lns. 1-11; and p. 478, rt. col., lns. 8-28).

In the first analysis, a methanol injection solvent (*i.e.*, methanol/H_2O) and a methanol/H_2O/CO_2 injection solvent were compared with regard to separation of a coal tar sample. (P. 479, left col., "Results and Discussion"; rt. col., first full para.; and p. 480 "Figure 1"). "No difference" and "no observable effect" were seen between the methanol/H_2O and the methanol/H_2O/CO_2 mixtures. (P. 479, rt. col., first full para.). In the fourth analysis, "probucol" was separated from related compounds using the EF mobile phase "across one column." (P. 482, left col., lns. 34-36).

Finally, in the fifth analysis, a mixture of vitamins was separated in a single column with the EF mobile phase conditions. Lee emphasized that the separation of vitamin D_3 and vitamin D_2 from the mixture of fat soluble vitamins was attributable only to the "reversed-phase conditions":

> Reversed-phase HPLC, unlike normal phase HPLC and SFC, is ***the only technique*** by which vitamins D_2 (ergocalciferol), and D_3 (cholecalciferol) can be resolved. Figure 5 shows a separation of fat-soluble vitamins with the EF mobile phase conditions across one column. Baseline resolution for most of these compounds is achieved in under 20 min. ***Partial resolution of vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) is also achieved. This is attributed to the separation taking place under reversed-phase conditions***. (P. 482, rt. col., lns. 2-13). (Emphasis added).

SUMMARY OF THE ARGUMENT

The Examiner has failed to meet her burden of making a *prima facie* case of obviousness. Zhang has no "deficiency" that needed to be "alleviated," and to the

extent the Examiner conceded factual deficiencies in the rejection, the Examiner has not provided any reason why one would have picked and combined the abstracts as the Examiner did. The Examiner began her analysis with the presumption that the claimed process was obvious and improperly placed the initial burden on the applicant. Nothing in the disclosures of the abstracts or in the Examiner's reasoning would have suggested a process as claimed. The Examiner has not even acknowledged, much less dealt with, the Lee article or its disclosure that leads away from Zhang. The Examiner never even addressed, much less demonstrated, that dependent claims 6, and 7 would have been obvious, and the Examiner's summary "within the skill" and "no problem" arguments are insufficient to render claim 4 obvious.

ARGUMENT

POINT I

ZHANG HAS NO "DEFICIENCY" THAT NEEDED TO BE "ALLEVIATED," AND TO THE EXTENT THE EXAMINER CONCEDED FACTUAL DEFICIENCIES IN THE REJECTION, THE EXAMINER HAS NOT PROVIDED ANY REASON WHY ONE WOULD HAVE PICKED AND COMBINED THE ABSTRACTS AS THE EXAMINER DID

Although, as demonstrated further below, the rejection over the combination of the Zhang and Lee abstracts without consideration of the totality of the teachings of the corresponding Lee publication was improper, because the Examiner failed to meet her burden even with regard to only the two isolated abstracts, we initially demonstrate that the rejection over the abstracts should be reversed even without consideration of the Lee publication.

As is clear from the rejection, the Examiner relied on Zhang for disclosing irradiation of a vitamin D_3 solution followed by separation of isomers in normal phase HPLC and on a silica column. (Paper No. 15, p. 3, lns. 1-12). According to the Examiner, the only element required by claim 1 and missing from Zhang, was a mobile phase containing either supercritical CO_2 or liquid CO_2. (*Id.*, at lns. 13-15). Although Zhang disclosed that the mobile phase for separating vitamin D_3 should be n-pentanol in hexane (*i.e.*, with no mention of liquid CO_2), the Examiner relied on Lee to "***alleviate this deficiency***" because, according to the Examiner, Lee disclosed the use of a methanol/H_2O/CO_2 mobile phase "in a column," and thereby satisfied all of the limitations required by the claims. (*Id.*, at p. 3, lns. 16-19). [Emphasis added].

The first error in the rejection, is that the Examiner did not identify any ***"deficiency"*** in Zhang's technique ***that needed to be "alleviated."*** Zhang's technique of employing an n-pentanol in hexane mobile phase worked for Zhang, and nothing in the disclosure of the Zhang abstract even suggests otherwise. Moreover, the Examiner has not provided any factual basis or cited any factual evidence to support the allegation that Zhang's technique had a "deficiency" that needed to be "alleviated."

Thus, to the extent the Examiner relied on a "deficiency" in the Zhang technique that needed to be "alleviated" to support the combination with the Lee abstract, the rejection is based on either a misinterpretation of Zhang, conjecture, or on facts within the personal knowledge of the Examiner. Regardless of which it is, each scenario provides a sufficient basis to reverse the rejection. *See Ex parte Porter*, 25 USPQ2d 1144, 1147 (BPAI 1992) (reversing a rejection based on a misinterpretation of fact) *Ex parte Levy*, 17 USPQ2d 1461, 1465 (BPAI 1990) (same); *Ex parte Natale*, 11

USPQ2d 1222, 1226 (BPAI 1989) (speculation not a substitute for a factual disclosure); *In re Pagliaro*, 210 USPQ 888, 892 (CCPA 1981) (same); 35 USC § 132 (The PTO must provide applicant with clear notice of the basis for each rejection); and *Ex parte Saceman*, 27 USPQ2d 1472, 1474 (BPAI 1993) (obviousness must be based on facts). And, to the extent the Examiner was relying on facts within her personal knowledge, should the rejection be maintained, the Examiner is hereby requested to make a showing in accordance with MPEP § 2144.03 and 37 CFR § 1.104(d)(2) to support the Examiner's "deficiency" allegation.

In addition, although an applicant is not required to guess at what the factual basis for a rejection is (*see, again,* 35 USC § 132), assuming, alternatively, that the Examiner was attempting to "alleviate" a "deficiency" in the factual disclosure of Zhang, as explained below, such factual deficiencies cannot be cured without providing the requisite suggestion or motivation, as required by § 103, to make a combination as claimed. The Examiner has fundamentally failed to meet that burden.

As is clear from the Paper No. 15 Office action, the Examiner summarily contended that "one skilled in the art would find ***ample motivation from the prior art*** to separate vitamin D derivatives as instantly claimed ***for the reasons cited above***." (Paper No. 15, p. 4, lns. 7-9). (Emphasis added). However, the only reason even arguably provided by the Examiner for picking and choosing the elements she combined, was that they exist. Specifically, the so-called "reasons cited above" were simply that the Zhang and Lee abstracts were from the "same field of endeavor," and that:

> Zhang *et al.* teaches irradiation technique and the purification on silica column (stationary phase) and Lee et

al., teaches the use of liquid CO_2 for separation. (Paper No. 15, p. 4, lns. 2-9).

In addition to the fact that Lee does not "teach the use of liquid CO_2" as the Examiner asserted but rather a "methanol/H_2O/CO_2" mobile phase, nothing in the Examiner's statement above provides or identifies any suggestion to make the combination the Examiner made. But it is well settled that, where, as here, the Examiner "asserts that there is an explicit or implicit teaching or suggestion in the prior art, [the Examiner] ***must indicate where such a teaching or suggestion appears in the reference***." *In re Rijckaert*, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993) (citing *In re Yates*, 211 USPQ 1149 (CCPA 1981)). [Emphasis added]. The Examiner failed to meet that burden, and for that further reason, the rejection should be reversed.

Moreover, merely advancing that the abstracts are in the "same field of endeavor," and are "obvious to combine" – which is not even the standard under §103, does not relieve the Examiner of her burden of demonstrating ***where*** in Lee there is a suggestion to choose the process components required by the claims, and ***why*** one would have chosen those components and incorporated them into a process as claimed. The kind of suggestion that would have "***strongly motivated***" one to pick either of the mobile phases recited by claim 1, and to make a process as claimed [*Ex parte Graselli*, 231 USPQ 393, 394 (Bd. App. 1983)], the type of motivation that would have "***impelled***" one to do so [*Ex parte Levengood*, 28 USPQ2d at 1300, 1302 (BPAI 1993)], and the type of suggestion that the selection and combination "***should***" be made [*Ex parte Markowitz*, 143 USPQ 303, 305 (Bd. App. 1964)]. But that is what a conclusion of obviousness requires. *See*, *Levengood*, 28 USPQ2d at 1302. The

Examiner has not even attempted to address these elements, and without these elements, obviousness cannot be established.

Because the basic elements necessary to support a rejection under § 103 have not been established, the rejection should be reversed.

Moreover, what is apparent, is that in formulating the rejection, the Examiner repeatedly employed the wrong legal standard, and merely relied on the fact that elements required by the claims were separately "known." For example, in addition to the quoted portions of the office actions noted above, in the Paper No. 10 Advisory action, the Examiner summarily contended that it "***would have been obvious*** who is familiar with the art ***because these techniques are well known***." (P. 2, para. Item No. 10). Contrary to the Examiner's "known" argument, no such rule of law exists, and the Examiner has not cited any authority for her *per se* rule of what is *prima facie* obvious. Of course, it is well settled that there are no *per se* rules of patentability (see MPEP § 2116.01 at 2100-52; and see also the Commissioner's Notice at 1184 OG 86), and as the Federal Circuit emphasized in *In re Rouffet*, 47 USPQ2d 1453, 1457 (Fed. Cir. 1998), that something was "known" *per se*, is insufficient to support a rejection under § 103:

> As this court has stated, "virtually all [inventions] are combinations of old elements." *Environmental Designes, Ltd. v. Union Oil Co.*, 713 F.2d 693, 698, 218 USPQ 865, 870 (Fed. Cir. 1983); see also *Richdel, Inc. v. Sunspool Corp.*, 714 F.2d 1573, 1579-80, 219 USPQ 8, 12 (Fed. Cir. 1983) ("Most, if not all, inventions are combinations and mostly of old elements."). Therefore, ***an examiner may often find every element of a claimed invention in the prior art. If identification of each claimed element in the prior art were sufficient to negate patentability, very few patents would ever issue. Furthermore, rejecting patents solely by finding prior art corollaries for the***

> ***claimed elements would permit an examiner to use the claimed invention itself as a blueprint for piecing together elements in the prior art to defeat the patentability of the claimed invention. Such an approach would be "an illogical and inappropriate process by which to determine patentability."*** *Sensonics, Inc. v. Aerosonic Corp.*, 81 F.3d 1566, 1570, 38 USPQ2d 1551, 1554 (Fed. Cir. 1996).

But merely identifying of "known" elements is all that the Examiner has done, and is all that the rejection is based upon.

Thus, because the Examiner has failed to provide the requisite suggestion for why one would have selected the references picked by the Examiner and combined them in a way that would have resulted in the claims on appeal, and because the Examiner has repeatedly relied on the wrong legal standard, the rejection of all claims should be reversed.

POINT II

THE EXAMINER BEGAN HER ANALYSIS WITH THE PRESUMPTION THAT THE CLAIMED PROCESS WAS OBVIOUS AND IMPROPERLY PLACED THE INITIAL BURDEN ON THE APPLICANT

As noted above, the reasoning the Examiner employed in making the rejection final is found in the Paper No. 15 Office action. In the Paper No. 15 Office action, the Examiner concluded that:

> ***It would have been obvious*** to one skilled in the art ***to combine the teachings of prior art*** supra ***to separate the vitamin D derivatives*** particularly when Zhang et al. teaches irradiation technique and the purification on silica column (stationary 4 phase) and Lee et al. teaches the use of liquid CO2 for separation. (Paper No. 15, p. 4, Ins. 1-6). (Emphasis added).

Although, as explained above, that analysis was improper and the rejection should be reversed in view of the above noted factual and legal deficiencies alone, whether or not it would have been obvious to combine the teachings of the abstracts "to separate the vitamin D derivatives," ***does not address and is irrelevant to whether the process claimed would have been obvious***, because the process claimed is not merely "separating vitamin D derivatives." The process claimed also requires ***normal phase*** chromatography – ***not reverse-phase*** chromatography – and either liquid CO_2 or supercritical CO_2 mobile phase. And, while the Examiner concluded that it would have been obvious to combine the abstracts to "separate the vitamin D derivatives," the Examiner did not conclude that ***the claimed process***, including all of its limitations, would have been obvious. Instead, when the Examiner finally addressed the claimed process, she began with the conclusion that the claimed process was obvious, and placed the initial burden on the applicant to show that it was not. That, was error.

Specifically, when the process claimed was finally addressed, the Examiner summarily concluded:

> ***Nothing unobvious*** to separate the vitamin D as instantly claimed by ***the process known in the art***, unless any unexpected results are seen. (Paper No. 15, p. 4, lns. 10-12). [Emphasis added].

Contrary to the Examiner's argument, the question under § 103 is not whether what is claimed is "unobvious." The question is whether one of ordinary skill in the art, with the references in hand, would have found that the precise process claimed is suggested by the references and, therefore, obvious. That is not semantics; that is the law. And the law does not place the burden on the applicant to show that what is

claimed is not obvious; the law places the burden on the Examiner to show that what is claimed ***is obvious***. *See Ex parte Obukowicz*, 27 USPQ2d 1063, 1065 (BPAI 1993) (and cases cited therein).

As is plainly apparent from the rejection, the Examiner began with the conclusion that the process claimed was not "unobvious," and placed the burden on the applicant to demonstrate that it was not. In doing so, the Examiner erroneously shifted the initial burden to the applicant and fundamentally failed to meet her burden.

Moreover, the Examiner's reasoning that the references are combinable "because they are from the same field of endeavor," is not a substitute for meeting her burden. Even when references are in related fields, the Examiner still has the burden of establishing (1) that there is a suggestion or motivation to combine the references relied upon, and (2) that the references, when so combined, contain the requisite suggestion and motivation that would have led one to combine the particular disclosure relied upon and to make the process or composition as claimed. *In re Dembiczak*, 50 USPQ2d 1614, 1617 (Fed. Cir. 1999).

At bottom, in view of all of the foregoing errors, it is apparent that the rejection is a classic case of using the claims as a guide, and attempting to knock down each of their limitations in isolation rather than considering the claims as a whole. Because the Examiner failed to meet her burden for these additional reasons, the rejection should be reversed.

POINT III

NOTHING IN THE DISCLOSURES OF THE ABSTRACTS OR IN THE EXAMINER'S REASONING WOULD HAVE SUGGESTED A PROCESS AS CLAIMED

Although it is believed that, for the reasons presented in Points I and II above and in Points IV and V below, appellant has no burden to demonstrate the unobviousness of the claimed process, out of an over-abundance of caution, we also demonstrate below that in view of the Zhang and Lee abstracts alone the process claimed would not have been obvious to one of ordinary skill in the art at the time the invention was made.

As noted above, Lee does not "teach the use of liquid CO_2" as alleged by the Examiner. What Lee discloses in the abstract, is the use of a "methanol/H_2O/CO_2 mobile phase," not merely a "liquid CO_2" mobile phase. But even the mere disclosure of a methanol/H_2O/CO_2 mobile phase does not replace the Examiner's burden of identifying ***where*** in Zhang or Lee there is a suggestion that would have led one to combine the Zhang and Lee abstracts at all, much less to combine them in a way that would have made the present claims obvious. And nothing in the disclosures of the abstracts would have suggested a process as claimed.

The Zhang abstract discloses an analysis of a vitamin D_3 solution by ***normal phase*** HPLC on a silica column and with an ***n-pentanol in hexane mobile phase***. The Lee abstract, on the other hand, discloses applications of ***reverse-phase*** HPLC employing samples of (1) coal tar, (2) fat soluble vitamins, and (3) probucol and related compounds, and while not actually mentioning whether vitamin D_3 was included in the fat soluble vitamins, also mentions cholecalciferal, which is vitamin D_3, at "IT".

The Lee abstract also discloses that Lee's ***reverse-phase*** method employed a ***methanol/H_2O/CO_2 mobile phase***.

Thus, based on those disclosures, one of ordinary skill in the art with the two abstracts in hand, would have been presented with choices between separating (1) a coal tar sample, or (2) probucol and related compounds, or (3) fat soluble vitamins generically, or (4) vitamin D_3. Nothing in the combined disclosures of the abstracts suggests separating vitamin D_3 over coal tar, or probucol and related compounds, or fat soluble vitamins generally.

One of ordinary skill in the art with the two abstracts in hand, also would have been presented with choices between (1) normal-phase HPLC, and (2) reverse-phase HPLC. Again, nothing in the combined disclosures of the abstracts suggests normal-phase HPLC over reverse-phase HPLC. To the contrary, what is suggested is that if one wanted to separate vitamin D_3 with a mobile phase containing liquid CO_2 (*i.e.*, a methanol/H_2O/CO_2 mobile phase), ***the separation should be done by reverse-phase HPLC, <u>not</u> normal phase HPLC***. But that, is not what is claimed. And that is not suggestive of what is claimed.

One of ordinary skill in the art with the two abstracts in hand, also would have been presented with choices between (1) an n-pentanol in hexane mobile phase, and (2) a methanol/H_2O/CO_2 mobile phase. And again, nothing in the combined disclosures of the abstracts suggests deviating from Zhang at the point of the mobile phase and changing only Zhang's mobile phase in any way. But the Examiner ignored Zhang's explicit teaching to use an n-pentanol in hexane mobile phase when separating

a vitamin D_3 mixture by normal phase HPLC, and instead chose to select "liquid CO_2" from Lee even though, as explained above, Lee does not "teach the use of liquid CO_2."

Again, nothing in the combined disclosures of the abstracts suggests any reason to have deviated at that point and to have made the selection the Examiner made, and neither does the statement of the rejection. And change for the sake of change is not what one of ordinary skill in the art seeks to do. *See Standard Oil Co. v. American Cyanamid Co.*, 227 USPQ 293, 297-98 (Fed. Cir. 1985) ("One of ordinary skill in the art ***follows conventional wisdom and does not innovate***").

At bottom, there is no mention in either abstract that any benefit may be achieved by proceeding in a contrary fashion to the express teachings of either abstract. There is not even the hint of suggestion for why one would have deviated from Zhang's teachings on the one hand, or from Lee's teachings on the other hand. And most importantly, there is no suggestion in the combined abstracts to have proceeded as claimed. There is only a teaching ***not to deviate*** from their express disclosures.

Because the abstracts would not have suggested deviating from Zhang's or Lee's express teachings in any way, the combination of Zhang and Lee would not have suggested the claimed process.

In addition to all of the foregoing, the Examiner did not explain ***why*** one would have deviated from the clear and explicit teaching of Lee to use ***reverse-phase*** HPLC with a methanol/H_2O/CO_2 mobile phase to separate fat soluble vitamins such as vitamin D_3. Nor did the Examiner explain ***why*** one would have deviated from the Zhang method at all, much less ***only*** at the point of the mobile phase. The Examiner did not

make any factual findings regarding those analyses, and without any such findings, obviousness cannot be established. *See Ex parte Saceman*, 27 USPQ2d 1472, 1474 (BPAI 1993) (Obviousness "***must***" be based on facts). *In re Freed*, 165 USPQ 570, 571-72 (CCPA 1970 ("Cold hard facts"). When a rejection under § 103 is not based on facts, it can not stand. *Ex parte Porter*, 25 USPQ2d at 1147 (BPAI 1992).

Moreover, even if there were reason – based on factual evidence – to deviate from Lee's explicit teachings to separate vitamin D_3 using ***reverse-phase*** HPLC, or to deviate from the Zhang method at the point of the mobile phase, and even if the Examiner had met her burden of providing factual evidence for ***why*** such a deviation was suggested, the Examiner still did not even propose what the resulting hypothetical process would be. That is, the Examiner did not explain whether one would have replaced Zhang's n-pentanol in hexane mobile phase with only the so-called "liquid CO_2," or whether one would have added the "liquid CO_2" to Zhang's n-pentanol in hexane mobile phase, or whether one would have combined Zhang's n-pentanol in hexane mobile phase and Lee's methanol/H_2O/CO_2 mobile phase, or whether one would have replaced Zhang's n-pentanol in hexane mobile phase entirely with Lee's methanol/H_2O/CO_2 mobile phase. That, also, was the Examiner's burden, and, again, a mere allegation that the elements were "known," cannot satisfy that burden.

That omitted analysis was essential to the rejection, because before the Board can assess whether a combination proposed by an Examiner would have been suggested and obvious to one of ordinary skill in the art, the Board needs to know, precisely, what the proposed modifications of the references are and what the ultimate

combination is. That is, the Board needs to make the determination whether one of ordinary skill in the art would have found a suggestion to change in any way (*e.g.*, add to or make a wholesale substitution of) Zhang's n-pentanol in hexane mobile phase, and if so, how one would have employed Lee's methanol/H_2O/CO_2 mobile phase or the so-called "liquid CO_2," in making that change. The Examiner failed to meet that burden too, and because the Examiner failed to meet that burden, the rejection should be reversed for this additional reason.

POINT IV

THE EXAMINER HAS NOT EVEN ACKNOWLEDGED, MUCH LESS DEALT WITH, THE LEE ARTICLE OR ITS DISCLOSURE THAT LEADS AWAY FROM ZHANG

A. The Examiner Should Have Considered The Lee Article That Corresponds To The Lee Abstract

As is fundamental, one of ordinary skill in the art is aware of and considers the art as a whole. *In re Dow Chemical*, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988); and *In re Jezel*, 158 USPQ 98, 99-100 (CCPA 1968) (all references are part of the scope and content of the prior art). Here, the rejection is based upon two abstracts without any reference to a corresponding article for either abstract. And although the complete Lee article that the abstract was cropped from made of record in October of 2000 and was repeatedly relied upon by the applicant, the Examiner never addressed the disclosure of the Lee article. Instead, the Examiner repeatedly relied solely on the Lee abstract, and only in the Final action did the Examiner attempt to respond to applicant's reliance on the Lee article. That, again, was error.

As a unanimous panel of the Board explained in *Ex parte Gavin*, 62 USPQ2d 1680, 1683-84 (BPAI 2001) (unpublished), it is improper to rely on an abstract where the corresponding full document is available:

> The Board of Patent Appeals and Interferences continues to have recurring problems in resolving ex parte appeals which come before it. ***One continuing recurring problem is the citation and reliance by examiners on abstracts, without citation and reliance on the underlying scientific document.***
>
> In this appeal, the examiner relied upon abstracts of two published Japanese patent applications without referring to translations of the underlying applications. An abstract and the underlying document of which it is a summary are distinct documents. In a rejection, an abstract stands on its own – it does not incorporate by reference any disclosure of the underlying document. ***Abstracts are often not written by the author of the underlying document, and may be erroneous or misleading – in virtually all cases, they are incomplete.***
>
> * * * *
>
> ***Generally an abstract does not provide enough information to permit an objective evaluation of the validity of what it describes. Thus, an abstract is even less reliable a basis to extrapolate the alleged teachings of the underlying document to different circumstances.*** Abstracts function to alert a reader to disclosures of possible interest. They are little more reliable than headlines or brief newspaper articles.
>
> ***Citation of an abstract without citation and reliance on the underlying scientific document itself is generally inappropriate where both the abstract and the underlying document are prior art.*** It is our opinion that a proper examination under 37 CFR Section 1.104 should be based on the underlying documents and translations, where needed. Accordingly, the preferred practice is for the examiner to cite and rely on the underlying document.

The Lee article has long been available to the Examiner for consideration, and it contains disclosure that refutes the rejection. The Examiner's repeated failure to address its disclosure formally on the record before making and maintaining the rejection – because a rejection would not withstand the combination of Zhang and the Lee article – demonstrates that fact.

For this further reason the rejection was fundamentally flawed and should be reversed.

B. Lee Explicitly Leads Away From Using The Zhang Normal Phase HPLC

As is fundamental, "[a] prior art reference must be considered in its entirety, *i.e.*, as a whole, ***including portions that would lead away from the claimed invention.***" (Underline original, bold emphasis added). *See* MPEP § 2141.02 at 2100-95. A primary reference that "teaches away" from a secondary reference is sufficient to show that one would not have combined the two references. *See* MPEP § 2145 at 2100-123 ("It is improper to combine references where the references teach away from their combination.").

As noted above, the Lee article [Exhibit 1] discloses various analyses on various samples, and ***all were*** performed by a ***reverse-phase*** HPLC process. And with particular regard to resolving vitamin D_3 from a mixture of other fat soluble vitamins that included vitamin D_2, Lee explicitly teaches that if one wants to resolve vitamin D_3 with a mobile phase containing CO_2, "reverse-phase conditions" is the way it must be done:

> Reversed phase HPLC, unlike normal phase HPLC and SFC, is ***the only technique*** by which vitamins D_2

> (ergocalciferol), and D_3 (cholecalciferol) can be resolved. Figure 5 shows a separation of fat-soluble vitamins with the EF mobile phase conditions across one column. Baseline resolution for most of these compounds is achieved in under 20 min. ***Partial resolution of vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) is also achieved. <u>This is attributed to the separation taking place under reversed-phase conditions</u>***. (P. 482, rt. col., Ins. 2-13). (Emphasis added).

That, is what one of ordinary skill in the art would have taken away from Lee. And that, is the opposite of what Zhang teaches, and is not what is claimed. That is not suggestive of what is claimed.

In this regard, we note that Lee was published in 1995, and presumably had Zhang (which was published in 1990) before them. Thus, considering the state of the art as reflected by the earlier-in-time Zhang and then the later-in-time Lee, one of ordinary skill in the art, in view of the totality of the factual evidence, would have followed the teachings of Lee and proceeded with a reverse-phase HPLC process, not a normal phase HPLC. The Examiner has not contended otherwise and, as noted above, has not offered any reason whatsoever for why one would have deviated from Lee's reverse-phase HPLC, or combined the two references to arrive at a process as claimed.

Although these arguments and evidence have been repeatedly advanced and relied upon by the applicant, the only response to them that the Examiner ever offered was in the Final action, where the Examiner summarily stated:

> "instant invention does not give any separation of vitamin D_3 and vitamin D_2 by normal phase HPLC, rather it is claiming separation of vitamin D_3 with other components such as dehydrocholesterol, lumisterol and others." (Paper No 17, p. 2, ¶ 2, Ins. 2-8).

The Examiner's response misses the point. Whether or not Lee discloses a process for separating vitamin D_2 and vitamin D_3, ignores the fundamental principle that references are to be considered in their ***entirety*** including teachings that would have suggested proceeding in a contrary direction to what is claimed. The Lee abstract and Lee article both lead one, who wanted to separate vitamin D_3 from a mixture, to use ***reverse-phase*** HPLC. The Lee reverse-phase HPLC process not only gives separation of vitamin D_3 from a mixture of other vitamins, it also is the "only technique by which vitamins D_2 (ergocalciferol), and D_3 (cholecalciferol) can be resolved." (P. 482, rt. col., Ins. 2-13). When that disclosure is considered in light of Zhang who sought to separate a vitamin D_3 solution into various isomers, one of ordinary skill in the art, seeing the enhanced resolution of vitamins provided by the Lee process, particularly vitamin D_2 and vitamin D_3, would have followed Lee's ***reverse-phase*** process, and not proceeded as claimed. That is the point, and the Examiner still has not dealt with it.

The Lee abstract and Lee article both lead away from what is claimed, and the Examiner has not provided any reason why one would not have followed Lee's direction, much less proceeded in the direction the Examiner did in making the rejection. The only suggestion to have proceeded as claimed, came from appellant's claims. And nothing in the rejection demonstrates otherwise.

Thus, for this further reason, the rejection of all claims should be reversed.

POINT V

THE EXAMINER NEVER EVEN ADDRESSED, MUCH LESS DEMONSTRATED, THAT DEPENDENT CLAIMS 6, AND 7 WOULD HAVE BEEN OBVIOUS, AND THE EXAMINER'S SUMMARY "WITHIN THE SKILL" AND "NO PROBLEM" ARGUMENTS ARE INSUFFICIENT TO RENDER CLAIM 4 OBVIOUS

As the Final action and the Office action that preceded it reflect, the Examiner never even addressed, much less demonstrated, that claims 6 and 7 would have been obvious. (*See* Paper Nos. 15 and 17). That was error, because for each claim, the Examiner was required to demonstrate ***where*** in the references "by page and line" there is a disclosure of each of the limitations required by the claim (see *Chiong v. Roland*, 17 USPQ2d 1541, 1543 (BPAI 1990)), and explain ***why*** one would have been led to a combination of elements arranged as in the claims. An applicant for a patent has ***no burden*** until the Examiner has met her burden.

For this reason, as a matter of law, the Examiner has failed to meet her burden with respect to claims 6 and 7, and the rejection of those claims should be reversed.

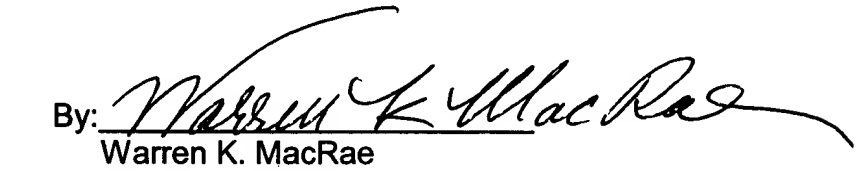
In addition, the sole arguments presented by the Examiner with respect to claim 4, was that it was within "the skill in the art," and that it would have been "no problem" for one to figure out the particular parameters of the limitations recited in claim 4. (Paper No. 15, p. 4, lns. 16-21). The rejection of claim 4 also should be reversed because that standard has been flatly rejected by the Board. *See, e.g., Ex parte Levengood*, 28 USPQ2d 1300, 1301, and 1302 (BPAI 1993) (that which is within the skill of one in the art is not synonymous with obviousness). Similarly, "no problem" is not the standard under § 103 and cannot release the Examiner from her burden.

And no case supports the Examiner's novel standard for § 103. Because those were the only arguments advanced by the Examiner with regard to claim 4, and because those standards have been rejected, the rejection of claim 4 also should be reversed.

CONCLUSION

For all of the foregoing reasons, it respectfully is submitted that the Examiner has failed to make out a *prima facie* case of obviousness and hence the rejection of claims 1-8 should be reversed.

Respectfully submitted,

By: 

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APPENDIX

1. A process for the isolation of vitamin D_3 or previtamin D_3 from a mixture containing vitamin D_3 or previtamin D_3, which process comprises separating the vitamin D_3 or previtamin D_3 by a normal phase column chromatographic technique, wherein a mobile phase of the chromatography comprises supercritical carbon dioxide or liquid carbon dioxide.

2. A process according to claim 1, wherein the mobile phase further comprises a modifier.

3. A process according to claim 1, wherein a silica gel is used as the stationary phase.

4. A process according to claim 1, wherein the silica gel is in the form of homogeneously packed, spherical particles having a particles size of about 5 to 25 μm.

5. A process according to claim 1, wherein a reaction mixture synthetically produced by irradiation is used as a mixture of vitamin D_3 isomers.

6. A process according to claim 1, which is carried out in the temperature range from about 30°C to about 60°C and in the pressure range from about 7.0 to about 15.0 MPa.

7. A process according to claim 2 wherein the carbon dioxide is supercritical carbon dioxide.

8. A process according to claim 2 wherein the carbon dioxide is liquid carbon dioxide.

Applications of Reversed-Phase High Performance Liquid Chromatography Using Enhanced-Fluidity Liquid Mobile Phases

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Abstract. Enhanced-fluidity liquid mobile phases (methanol/H_2O/CO_2) were used as eluents in reversed-phase HPLC. The low pressure drop across the column allowed serial connection of micro-scale columns to achieve the efficient separation of a coal tar sample. Other applications such as the separation of fat soluble vitamins and probucol and related compounds are shown.

Key words: *HPLC, enhanced-fluidity, viscosity, pressure drop*

INTRODUCTION

Supercritical fluids have markedly lower viscosities than those of liquids. For example, the viscosity of supercritical CO_2 at 40°C and 136 atm is 0.065 cP [1] compared to 0.35, 0.56, and 0.89 cP for acetonitrile, methanol, and water, respectively, at 25°C and ambient pressure. The low viscosities of supercritical fluid mobile phases result in increased solute diffusion, faster speed of analysis, and lower pressure drops across the chromatographic column in SFC than in HPLC. The low column pressure drop has facilitated the use of long and/or coupled columns in packed-column SFC to increase the total number of theoretical plates for separation of complex mixtures [2–4].

We have demonstrated the use of enhanced-fluidity (or low viscosity) liquid mobile phases as eluents in reversed-phase HPLC [5, 6]. We defined an enhanced-fluidity mobile phase as a common HPLC eluent to which high proportions of a low viscosity liquid, such as CO_2, has been added [7]. The previous publications demonstrated increased speed-of-analysis, chromatographic efficiency and decreased pressure drop across the chromatographic column in HPLC when enhanced-fluidity eluents were used. Herein, we demonstrate that the low pressure drop caused by the use of an enhanced-fluidity mobile phase can allow ready serial coupling of columns. These coupled columns were applied to the separation of a complex mixture—a coal tar standard. We also show additional applications such as the separation of fat-soluble vitamins and the separation of pharmaceuticals, such as probucol, and some closely related compounds with an enhanced-fluid mobile phase.

MATERIALS AND METHODS

The methanol/H_2O/CO_2 mixtures were prepared using two ISCO LC-2600 high-pressure syringe pumps. A known volume of a methanol/H_2O mixture at a composition of 0.70/0.30 mole fraction was placed in one pump. Liquid CO_2 at 136 atm and ambient temperature was held in another pump. Using the known density of CO_2 at these conditions, the appropriate volume of CO_2 was delivered to the pump holding the methanol/H_2O mixture. The methanol/H_2O/CO_2 mixture was then pressurized to 204 atm and allowed to equilibrate at ambient temperature for at least 12 h to ensure complete mixing of the solution.

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All experiments described in this article were performed using a 0.70/0.30 mole fraction methanol/H_2O mixture or a 0.49/0.21/0.30 mole fraction methanol/H_2O/CO_2 mixture. For convenience, the methanol/H_2O mixtures at 26 and 60°C are designated as the RT (room temperature) and ET (elevated temperature) mobile phases, respectively. The 0.49/0.21/0.30 mixtures at 26 and 60°C are designated as EF (enhanced-fluidity) and ET-EF (elevated temperature—enhanced-fluidity) mobile phases, respectively.

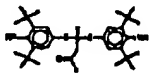
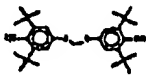
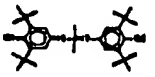
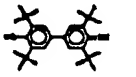
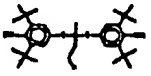
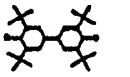
The chromatographic system consisted of an ISCO LC-2600 syringe pump (ISCO, Lincoln, NE), a Valco W-series high pressure injection valve with an injection volume of 200-nL (Valco Instruments, Houston, TX), BDS Hypersil C18, 150-mm × 1-mm columns packed with 5-μm diameter particles (Keystone Scientific, Bellefonte, PA) and a Spectra-Physics UV2000 UV/vis absorbance detector equipped with a capillary flow cell (model 9550-0155). The oven was that of a Carlo Erba Fractovap 4160 gas chromatograph. The mobile phase was preheated for the elevated temperature experiments by placing a 2-m length of 1/16-in i.d. stainless steel tubing inside the oven, after the syringe pump, and prior to the injector. The flow cell for detection was created by removing the polyimide coating from a 5-mm section of 100-μm i.d. fused silica tubing (Polymicro Technologies, Phoenix, AZ) and centering it in the capillary flow cell. The detector excitation wavelength was 254 nm for the polycyclic aromatic hydrocarbon test mixes and 230 nm for the probucol and vitamin test mixes. An Omega model PX931-5KSV pressure transducer (Omega Engineering, Stamford, CT) was placed in-line after the detector and before a post-detection restrictor. The flow control for the chromatographic system was maintained by a post detection restrictor that was an appropriate length of 20, 15, or 10-μm i.d. fused silica tubing (Polymicro Technologies, Phoenix, AZ). The outlet pressure of the column was monitored because the column pressure must be maintained above a minimum p to prevent the methanol/H_2O/CO_2 mixture from separating into two phases (liquid-gas). For example, a 0.506/0.218/0.276 mole fraction methanol/H_2O/CO_2 mixture at 59.7°C separates into two phases at pressures lower than 108.2 atm [8]. All experiments in this study were performed under conditions in which the methanol/H_2O/CO_2 mixture was a single liquid phase. The column inlet pressure was maintained at 204 atm throughout the chromatographic experiments except when using the RT mobile phaser with 4 columns in series. Under the RT conditions, a column inlet pressure of 320 atm was required to obtain the appropriate mobile phase linear velocity.

The primary chromatographic test mixture used in this study was a methanol solution containing 0.71 μL/mL benzene, 0.079 mg/mL naphthalene, 0.021 mg/mL anthracene, 0.082 mg/mL pyrene, 0.036 mg/mL benz[a]anthracene, 0.071 mg/mL benzo[e]pyrene, 0.039 mg/mL benzo[a]pyrene, and 0.046 mg/mL benzo[ghi]perylene. A Supelco test mix of 16 polyaromatic hydrocarbon compounds was also used (catalog #4-8905). This test mix was received in a methylene chloride/benzene solvent which was evaporated to dryness and replaced with methanol. The final concentration of each individual component of this test mix was 0.030 mg/mL.

A standard test mixture of vitamins was a methanol solution containing 0.02 mg/mL trans-retinol (vitamin A), 0.14 mg/mL butylated hydroxytoluene (BHT), 0.21 mg/mL trans-retinal (vitamin A aldehyde), 0.15 mg/mL ergocalciferol (vitamin D_2), 0.16 mg/mL cholecalciferol (vitamin D_3), 0.17 mg/mL retinol acetate (vitamin A acetate), 0.13 mg/mL ±α-tocopherol (vitamin E), 0.10 mg/mL ±α-tocopherol acetate (vitamin E acetate), and 0.12 mg/mL vitamin K_1. All vitamin standards were purchased from Sigma Chemical Company (St. Louis, MO) with the exception of retinol which was purchased from Aldrich (Milwaukee, WI). A mixture of probucol and related analogues was also characterized. The test mixture of these was a methanol solution containing 0.12 mg/mL compound 1, 0.084 mg/mL compound 2, 0.11 mg/mL probucol, 0.10 mg/mL compound 4, 0.096 mg/mL compound 5, and 0.11 mg/mL compound 6. The compound number and structures are shown in Table I. The probucol and analogue standards were donated by Marion Merrell Dow, Inc. (Cincinnati, OH). SRM 1597, a complex mixture of PAH isolated from coal tar, was obtained from The National Institute of Standards and Technology (Gaithersburg, MD).

Data were collected on an IBM AT-compatible computer using a data collection program written in our lab with ASYST 2.1 (Macmillan Software Company, New York, NY). Theoretical plates were determined by fitting a

Table I. *Number and structure of compounds in the probucol and analogue standard.*

Compound	Structure
1	
2	
3 Probucol	
4	
5	
6	

Gaussian peak to the experimental data using PeakFit 3.0 (PeakFit Analysis Software, Jandel Scientific, San Rafael, CA).

RESULTS AND DISCUSSION

Injection Profile. Methanol was chosen as the injection solvent because it is the main constituent of the mobile phase and all of the test compounds were readily soluble in it. An extra peak was always observed in the chromatograms. This peak was also a negative deflection from the baseline. This type of "system peak" is expected when a mixed mobile phase is used and the injection solvent is not the same composition as the eluent [9]. Therefore, with methanol as the injection solvent and methanol/H_2O/CO_2 mixtures as the eluents, system peaks would be expected. As long as the system peak and the analyte peaks do not overlap, the system peak does not effect the retention of the analytes under investigation. To test whether the negative peak was a system peak, the methanol/H_2O/CO_2 mixture was used as an injection solvent for one study.

The sample was injected with the pressurized methanol/H_2O/CO_2 mobile phase by splitting mobile phase eluting from the pump with a three-way valve. The majority of the mobile phase was delivered through the tubing, injector, column, detector, restrictor in that order as usual. The remaining mobile phase split at the three-way valve was delivered to a 1.2 mL extraction cell containing the sample solutes. The sample to be injected was prepared by delivering 1.2 mL of the chromatographic test mix to the extraction cell with a glass pipet and evaporating the methanol sample solvent with a stream of nitrogen. This process was repeated once so that the total volume of test mix delivered to the cell and evaporated was ca. 2.4 mL. The cell containing the sample solutes was then put in line between the three-way valve and the injector. The mobile phase solvated and pressurized the contents of the extraction cell. The cell and sample loop were maintained at the head pressure of the system by a valve positioned after the injector. A restrictor directly after the valve was created with a length of 5 μm id fused silica. By opening the post injector valve, sample was allowed to flow from the extraction cell and fill the sample loop. Using this system, triplicate injections of the 16-component standard PAH mixture were made. This was compared to triplicate injections of the 16-component PAH standard mixture using a conventional syringe guide and waste line respectively.

Figure 1 shows chromatograms using methanol as the injection solvent and using the 0.49/0.21/0.30 mole fraction methanol/H_2O/CO_2 mobile phase as the injection solvent. This comparison shows no difference in terms of efficiency (plate height) or selectivity (k') for the separation of the 16-component PAH test mix. However, the phase non-equilibrium introduced by using methanol as the injection solvent is shown in Figure 1A. A large positive deflection followed by a large negative deflection in the signal was observed when using methanol as the injection solvent. Only a small negative deflection in the baseline when using the mobile phase as the sample solvent is observed. The small negative deflection is attributed to methanol that was not completely evaporated under the nitrogen stream. In addition, some of the more volatile low molecular weight components (benzene, naphthalene, acenaphthalene, and fluoranthene) were lost when the methanol was evaporated under the nitrogen stream. Because there was no observable effect on the chromatographic performance when using methanol as the injection solvent, besides the observed system peak, methanol was used as the injection solvent for all other experiments in this study.

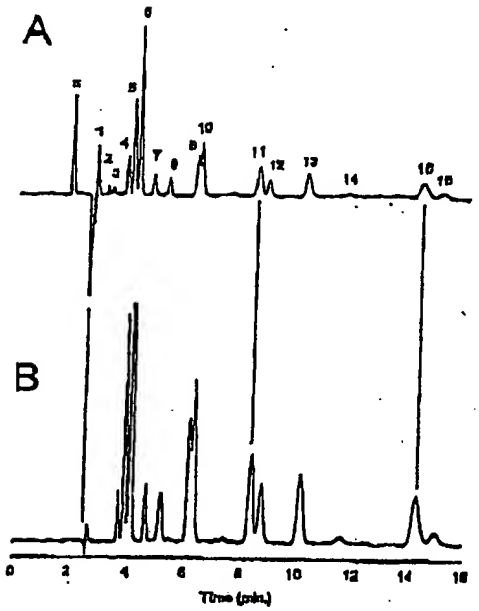


Figure 1. *Chromatograms with the EF mobile phase at 204 atm using (A) methanol and (B) 0.49 / 0.21 / 0.30 mole fraction methanol / H_2O / CO_2 mixture (EF mixture) as the injection solvent. (s) solvent, (1) benzene, (2) naphthalene, (3) acenaphthalene, (4) fluorene, (5) phenanthrene, (6) anthracene, (7) fluorene, (8) pyrene, (9) benz [a]anthracene, (10) chrysene, (11) benzo[b] fluoranthene, (12) benzo[k]fluoranthene, (13) benzo[a]pyrene, (14) dibenzo[a,h]anthracene, (15) indeno[1,2,3-cd]pyrene, (16) benzo[ghi]perylene.*

Pressure drop. Table II shows the pressure drop across the chromatographic system and the average number of theoretical plates for peaks 3–7 in Figure 1 at the 4 mobile phase conditions defined previously. The data were obtained at approximately the same linear velocity for 1 column and 4 columns in series. The data in Table I show the pressure drop across 4 chromatographic columns in series is roughly four-fold the pressure drop of one column for each of the 4 mobile phase conditions studied. The pressure drop across the column decreases in the following order of mobile phase conditions: methanol/H_2O at room temperature > methanol/H_2O at elevated temperature > enhanced-fluidity mixture at room temperature > enhanced-fluidity mixture at elevated temperature. Darcy's law [10, 11] describes the relationship between pressure drop, ΔP, column length, L, mobile phase viscosity, η, average linear velocity, $\langle u \rangle$, and particle diameter, d_p, in porous beds, such as chromatographic columns:

$$\Delta P = \frac{\phi \eta \langle u \rangle L}{d_p^2}$$

where ϕ is the dimensionless flow resistance parameter which typically has values in the range of 500–1000. The data in Table I are consistent with Darcy's law in that a linear increase in pressure drop with column length is predicted and observed. This expression and the data in Table I show the diminished pressure drop obtained by elevating the temperature of the methanol/H_2O mobile phase from 26–60°C, or by adding CO_2 to the mixture.

When the RT mobile phase with 4 columns at a linear velocity of 0.189 cm/s was used, the observed pressure drop across the chromatographic system was 314.2 atm (4617 psi). This pressure approaches the maximum pressure limits for many HPLC components currently in use. For example, the Valco injection valve

Table II. *Variation in column pressure drop and efficiency with mobile phase conditions and column length.*

One column	Mobile phase condition	Linear velocity (cm/s)	ΔP (atm)	N_{3-7} (plates)	N_{3-7} (plates/m)
	RT	0.196	81.3	7,618	50,787
	ET	0.204	49.2	8,880	59,200
	EF	0.207	39.2	9,955	66,367
	ET-EF	0.200	24.4	11,068	73,787
Four columns					
	RT	0.189	314.2	30,290	50,483
	ET	0.199	148.7	40,262	67,103
	EF	0.189	144.8	44,124	73,540
	ET-EF	0.182	81.9	47,948	79,913

used in this study has a maximum pressure rating of 340.2 atm (5000 psi). Therefore for the RT condition, the length of the chromatographic column cannot be extended significantly if one wishes to work at this linear velocity and conversely the linear velocity cannot be increased significantly using 4 columns in series.

Figure 2 compares a separation of PAH standards with the RT mobile phase on one column to that with the ET-EF mobile phase on 4 columns in series. The separations were both performed at approximately the same linear velocity, pressure drop, and analysis time. Retention is decreased with ET-EF mobile phase but the average number of theoretical plates for peaks 3–7 was more than six-fold greater for the ET-EF separation than for the RT separation. The dramatic increase in theoretical plate number can be attributed to several factors. The four-fold increase in column length accounts for ca. 64% of the total plate number for the ET-EF separation, leaving a 36% increase in plate number attributable to a combination of a shift in the optimum velocity to higher linear velocities, a decrease in the slope of the mass transfer region of the van Deemter curve, and decreased capacity factors (k'). See Reference 5 for a more detailed discussion of the relative contributions to plate height for the RT and ET-EF condition [5].

Figure 3 compares chromatograms of the coal tar (SRM 1597) sample separated at the same linear velocity with the EF mobile phase on 1 and on 4 columns. This comparison illustrates the improvement in efficiency when using 4 columns in series compared to one. However, at the same linear velocity, the analysis time is 4 times as long with 4 columns. The peaks in Figure 3 were tentatively identified by injecting the Supelco test mix that contained 16 polyaromatic hydrocarbon compounds immediately after collecting the coal tar chromatogram and superimposing these chromatograms on one another. In addition to the 13 compounds identi-

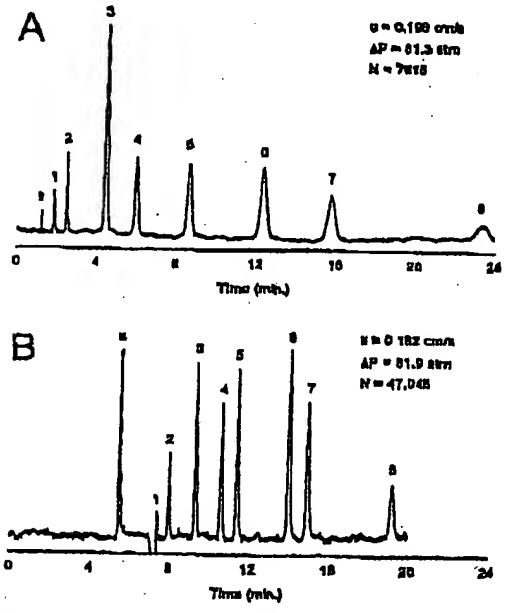


Figure 2. *Chromatograms at 204 atm with approximately the same column pressure drop but with (A) the RT mobile phase and 1 column; and (B) the ET-EF mobile phase and 4 columns. (s) solvent, (1) benzene, (2) naphthalene, (3) anthracene, (4) pyrene; (5) benz[a]anthracene, (6) benzo[e]pyrene, (7) benzo[a]pyrene, (8) benzo [ghi]perylene.*

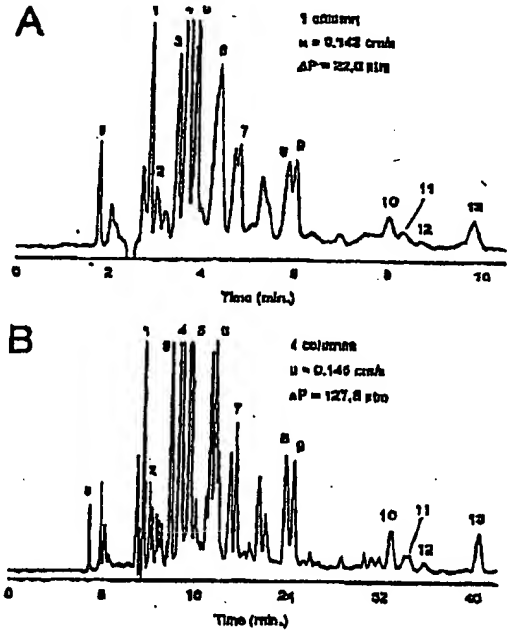


Figure 3. *Chromatograms of SRM 1597 coal tar standard using the EF mobile phase at 204 atm with (A) 1 column and (B) 4 columns. (s) solvent, (1) naphthalene, (2) acenaphthalene, (3) fluorene, (4) phenanthrene, (5) anthracene, (6) fluoranthene, (7) pyrene, (8) benz[a]anthracene, (9) chrysene, (10) benzo[b]fluoranthene, (11) benzo[k]fluoranthene, (12) perylene, (13) benzo[a]pyrene.*

fied in Figure 3, we identified 2 additional solutes, indeno[1,2,3-cd]pyrene and benzo[ghi]-perylene, at longer retention times; these peaks are not shown because the chromatograms were truncated to illustrate the increase in efficiency using the 4 columns in series. The identifications were further supported by referring to an article by Wise et al. and correlating our results with this extensive LC, GC, GC/MS analysis of the same sample [12].

APPLICATIONS

Probucol is a fat-soluble antioxidant that has been shown to lower serum cholesterol concentrations in humans [13]. The separation of some structural analogues of probucol were also investigated. Satonin and Coutant developed GC and HPLC methods for the analysis of the probucol and analogues [14]. The HPLC method was preferred because probucol decomposition occurred in GC analyses unless careful temperature control was used in the separation. The HPLC method of Satonin et al. was a reversed-phase separation using acetonitrile/hexane/0.1M ammonium acetate. As mentioned in previous sections of this paper, and demonstrated elsewhere [5], enhanced-fluidity liquids as eluents allow increased speed of analysis or increased efficiency due to the increased diffusion rates in the mobile phase. Therefore, a separation of compounds that would otherwise only be accomplished by elevated temperature HPLC can be accomplished by enhanced-fluidity LC at room temperature. Figure 4 is a separation of probucol and 5 structurally related compounds using EF mobile phase conditions across one column. The structures of the labeled compounds are listed in Table I. Baseline resolution was achieved for all compounds with the exception of compounds 2 and 3 in under 11 min. By contrast, peak 4 elutes at 43 min and peak 5 elutes at 75 min under RT conditions at the same linear velocity.

HPLC is the most commonly used technique for the analysis of fat-soluble vitamins. Several in-depth reviews on the chromatographic analysis of vitamins are available [15–17]. HPLC in both the reversed-phase and normal-phase modes is used for the analysis of fat-soluble vitamins. Packed column [18–20] and open tubular [21] supercritical fluid chromatography have also been investigated for the analysis of fat soluble vitamins. Reversed-phase HPLC is frequently used when simultaneous

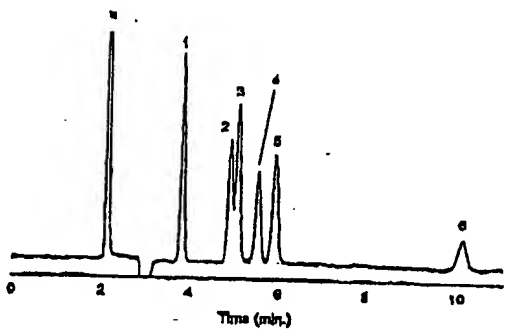


Figure 4. *Chromatogram of probucol and related compounds using the EF mobile phase at 204 atm.*

determination of several different vitamins in one chromatographic run is desired. Reversed-phase HPLC, unlike normal phase HPLC and SFC, is the only technique by which vitamins D_2 (ergocalciferol), and D_3 (cholecalciferol) can be resolved. Figure 5 shows a separation of fat-soluble vitamins with the EF mobile phase conditions across one column. Baseline resolution for most of these compounds is achieved in under 20 min. Partial resolution of vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) is also achieved. This is attributed to the separation taking place under reversed-phase conditions.

In summary, EF and ET-EF mobile phases are low viscosity mobile phases that are viable choices for extending the length of the chromatographic column to achieve a higher total number of theoretical plates for separations. These low viscosity mobile phases have previously been shown to increase the optimum lin-

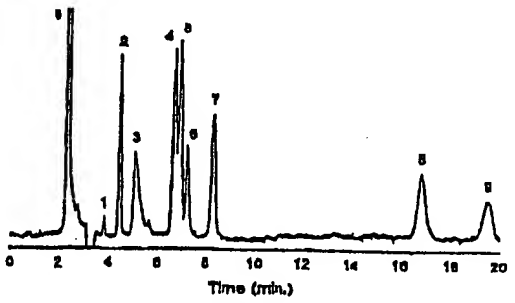


Figure 5. *Chromatogram of fat soluble vitamins using the EF mobile phase at 204 atm. (s) solvent, (1) trans-retinol, (2) BHT, (3) trans-retinal, (4) ergocalciferol, (5) cholecalciferol, (6) retinol acetate, (7) ±-tocopherol, (8) ±-tocopherol acetate, (9) vitamin K_1.*

ear velocity of the mobile phase and extend the operable linear velocity range resulting in shorter analysis times. In addition, the separations shown in this article demonstrate the applicability of enhanced-fluidity mobile phases in reversed-phase HPLC for a range of different types of compounds.

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